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This proposal is designed to further our understanding of the role of bone marrow derived endothelial precursor cells to mammary tumor angiogenesis, tumor growth and progression. We have designed a unique animal model system to carefully study the involvement of these cells in mammary tumor progression and tumor angiogenesis. The potential importance of these cells to cancer warrants the analysis of these bone marrow derived vascular cells to as many types of cancer settings as possible. Moreover, studies have shown that these endothelial precursor cells are regulated by estrogen in endometrial angiogenesis, suggesting they may have an important role in the vascularization of mammary tumors and may be affected by anti-estrogen therapy.

Specific Aims:

1. To determine the relative contribution of bone marrow derived endothelial cells to naturally occurring mammary tumors during stages of early, late and metastatic tumor growth.
2. To determine the impact of the angiogenic cytokines VEGF-A and PlGF on tumor recruitment of bone marrow derived endothelial cells
3. To determine if estrogen impacts on the recruitment of bone marrow derived endothelial cells to mammary tumors

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INTRODUCTION:

This grant proposes to investigate a novel but important area of tumor angiogenesis that has particular relevance to estrogen regulated tissues like the breast. . Recent studies of bone marrow derived cells have strongly suggested that endothelial cell precursor cells can be mobilized from the bone marrow and incorporated into sites of ongoing robust angiogenesis in adults (1). Aside from the female reproductive system, such sites are usually sites of pathological angiogenesis such as that involved in tumor growth. Recent findings demonstrate that bone marrow derived endothelial precursor cells can be rate limiting for the growth of some tumors (2). In addition, these same cells contribute to uterine angiogenesis in an estrogen regulated manner (3).

This proposal is designed to further our understanding of the role of bone marrow derived cells to mammary tumor angiogenesis, tumor growth and progression. We have designed a unique animal model system to carefully study the involvement of these cells in mammary tumor progression and tumor angiogenesis. The potential importance of these

cells to cancer warrants the analysis of these bone marrow derived vascular cells to as many types of cancer settings as possible. Moreover, studies have shown that these endothelial precursor cells are regulated by estrogen in endometrial angiogenesis, suggesting they may have an important role in the vascularization of mammary tumors and may be affected by anti-estrogen therapy.

Task 1. To determine the relative contribution of bone marrow derived endothelial cells to naturally occurring mammary tumors during stages of early late and metastatic tumor growth (months 1-18)
a. Using bone marrow transplanted MMTV PyV-MT mice, quantitate the relative contributions of bone marrow derived endothelial cell precursor cells at different tumor states
b. Examine circulating bone marrow precursor cells with multiple markers during different stages of tumor progression using real-time RTPCR.

Our initial goal in Task 1b, to characterize circulating precursor cells using different markers has made the most progress thus far. We were fortunate to have a pathology resident joint this effort and characterize VEGFR-2, VE-Cadherin, AC133 and Tie-2 in the blood of human breast cancer patients and to correlate this to tumor stage. This work resulted in a publication which is in press, attached in the appendix in galley form (4). In short, we were surprised to find the marker that most clearly correlates to breast cancer progression was Tie-2. Because of this we are using Tie-2 more often as a marker for these cells and have obtained Tie-2 lac Z mice to use in our mouse studies.

Task 2. To determine the impact of the angiogenic cytokines VEGF-A and on tumor recruitment of bone marrow derived endothelial cells (Months 6-24)

a. Make and test regulation in vitro of stable transfected mammary tumor cells isolated from PyV-MT tumors containing tetracycline regulated expression vectors containing murine VEGF-A and PlGF.
b. Examine the relative contribution of bone marrow derived endothelial cells in orthotopic tumors made from the cell lines described above.
c. Determine if tumor secreted cytokines from the cell lines described above influence the release of endothelial cell precursors into the blood stream

We are currently isolating the cell lines with regulated VEGF and PlGF expression and will begin to obtain data from this task this summer.

Task 3. To determine if estrogen impacts on the recruitment of bone marrow derived endothelial cells to mammary tumors (Months 12-36)

a. Examine the impact of tamoxifen on bone marrow derived endothelial cells in naturally forming tumors of the MMTVPyV-MT mice

b. Examine the impact of tamoxifen on the circulating endothelial cell precursor cells
c. Make estrogen independent sublines from the VEGF-A expressing cell lines made in Task 2.
d. Determine if estrogen or tamoxifen impacts on the incorporation of bone marrow derived endothelial cells to these tumors in high versus low VEGF-A expressing tumors

KEY RESEARCH ACCOMPLISHMENTS:

Optimization of protocol for real time RTPCR from human discarded blood
Characterization of RNA expression for VEGFR-2, Tie-2, VE-Cadherin and AC133 in peripheral blood of patients with carcinoma in situ, infiltrating carcinoma or non-tumor controls.
Writing and publishing paper reporting results
Transfection of stable cell lines with VEGF and PlGF
Purchase and breeding of Tie-2 lacZ mice for bone marrow transplant studies
Bone marrow transplant of lacZ bone marrow into Nude mice with VEGF and PlGF tumors
Harvest of tumors and immunohistochemistry to characterize the blood vessels
Investigation of Tie-2 signaling in endothelial cells to determine downstream pathways that could account for the Tie-2 association with circulating stem cells
Publication of our initial studies of Tie-2 signaling

REPORTABLE OUTCOMES:

Publication (s) attached to appendix

CONCLUSIONS:

The published research so far demonstrated that Tie-2 expression more than other markers of endothelial precursor cells correlates to breast cancer progression. This suggested that Tie-2 functions may be important for EPC function and stimulated us to turn our focus towards Tie-2 to a greater extent. To this end we have obtained a transgenic mouse with lac z expression driven by Tie-2 to use as a donor for bone marrow transplants and completed pilot experiments using these mice and our stable cell lines. In addition we have continued to understand how Tie-2 signaling may be important for these cells and identified the PI3Kinase/Akt signaling pathway as a major mediator of endothelial cell survival downstream of Tie-2. The latter findings were just published this past April.

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Research Paper

Blood Markers for Vasculogenesis Increase with Tumor Progression in Patients with Breast Carcinoma

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KEY WORDS

Tie-2, Vasculogenesis, Breast cancer, EPCs

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ABSTRACT

Recent studies show that AC133—a hematopoietic stem cell antigen, when coexpressed with endothelial markers, identifies a population of endothelial precursor cells (EPCs) in peripheral blood that function in tumor vasculogenesis in animals. Little is known about whether EPCs contribute to human tumor vasculogenesis. We attempted to determine if, through increased peripheral expression of AC133 or endothelial markers previously associated with EPCs,VEGFR-2 and Tie-2, we could detect an EPC response in the blood of patients with breast carcinoma. Thirty patients were segregated based on their breast biopsy histology into infiltrating carcinoma, DCIS and control groups. Using Real Time PCR, we measured the expression of the aforementioned markers in reverse transcribed RNA extracts from preoperative peripheral blood specimens. The cancer patients had significantly elevated Tie-2 expression with the highest levels associated with infiltrating carcinoma. Our data suggest increased circulating EPC markers in tumor patients, but further study of this cell population is needed to better define its role in tumor vasculogenesis.

INTRODUCTION

In order for tumors to be able to grow and ultimately metastasize, they need to acquire the ability to form their own vasculature. This is accomplished by recapitulating the normal vessel-forming process already present in the host. Two mechanisms are responsible for vessel formation: angiogenesis —the growth of new vessels as sprouts from pre-existing vessels, and vasculogenesis—the de novo formation of vessels via the recruitment of endothelial precursor cells (EPC) which are present in the bone marrow and in the circulation.[1,2] Relatively little is known about vasculogenesis since, until recently, there has been no morphologic, immunologic, or molecular property that could reliably identify this cell population. However, Peichev et al.[3] demonstrated that AC133 a hematopoietic stem cell antigen, when co-expressed with a vascular specific marker such as VEGFR-2 or Tie-2, identifies these cells.

Several studies have demonstrated that EPCs play a role in tumor vasculogenesis in experimental animal models,[4-7] but there is currently no published data on whether or not this process takes place in humans. In this study, we attempted to determine if, by virtue of increased expression of genes for AC133, VEGFR-2, and Tie-2 in breast carcinoma patients, we could demonstrate an increase in circulating EPC in these patients which would suggest that a similar mechanism of vasculogenesis may play a role in the vascularization of human breast cancer.

MATERIALS AND METHODS

We collected EDTA-anticoagulated whole blood specimens from women prior to undergoing excisional breast biopsies or mastectomies. The clinical histories were reviewed, and patients with a documented history of chemotherapy or radiation therapy within the previous 12 months, surgery or trauma within the previous two weeks, or any non-breast malignancy at any time were excluded. Patients with previous breast malignancies were excluded if the histology on the current specimen was benign. Based on the resulting histology, the patients were segregated into three groups: infiltrating carcinoma (ductal and/or lobular), ductal carcinoma in situ (DCIS), and a control group with benign histology. For the control group, we also obtained specimens from demographically matched patients with no history of malignancy who were scheduled to undergo non-tumor surgery.

RNA was extracted from the specimens using the Qiagen protocol. The extract was reverse-transcribed, and the optical density at 260nm was measured to establish that a sufficient quantity of cDNA was available for PCR analysis. For the PCR, we designed our own primers for actin, AC133, VEGFR-2, Tie-2, and VE-cadherin using the Primer Express Software from ABI (Applied Biosystems, Foster City, CA). These were designed to cross introns and tested to ensure that they do not amplify DNA. The sequences were as follows:

Table 1 Tumor Histology and Age of Patients Included in the Study

	Number of Patients	Mean Age
Infiltrating Carcinoma		
Ductal: 9	12	64
Lobular: 2		
Ductal & Lobular features: 1		
DCIS	7	57
Control group		
Benign breast histology: 4		
* Florid ductal hyperplasia		
* Intraductal papilloma		
* Microcysts with calcifications	11	59
* Fibroadenoma		
Non-breast surgery: 7		
* Cosmetic procedures 2		
* Carotid endarterectomy: 2		
* Hiatal hernia repair		
* Tubal ligation		
* Lithotripsy		
Total patients	**30**	**61**

Actin: forward 5′-CCTGGCACCCAGCACAAT; reverse 5′-GGGCCG-GACTCGTCATACT;

AC133: forward 5′-TGGATGCAGAACTTGACAACGT reverse 5′ATAC-CTGCTACGACAGTCGTGGT;

VEGFR-2: forward 5′-CACCACTCAAACGCTGACATGTA reverse5′-GCTCGTTGGCGCACTCTT;

Tie-2: forward 5′-GCTTGCTCCTTTCTGGAACTGT reverse 5′-CGC-CACCCAGAGGCAAT;

VE-cadherin: forward 5′-TTTCCAGCAGCCTTTCTACCA reverse: 5′-GGAAGAACTGGCCCTTGTCA;

The detection method used was SYBR Green. Forty cycles of Real Time PCR using the ABI Prism 7700 were performed in triplicate on each sample. RNA levels were quantified using standard curves set up from each primer pair. The ratio gene expression was internally controlled by comparison to actin, and an unpaired two-tailed student t test was performed to establish statistical significance of the data.

RESULTS

A total of 30 patients were studied (Table 1). These included 12 infiltrating carcinoma patients (9 ductal carcinomas, 2 lobular carcinomas, and 1 with combined ductal and lobular features), 7 DCIS patients, and 11 controls (4 breast patients with benign histology and 7 non-breast patients). All patients were analyzed for the expression of the aforementioned markers (Fig. 1). The proteins AC133, VEGFR-2 and Tie-2 have been previously associated with EPCs, while VE-Cadherin is a marker of fully differentiated endothelial cells. Mild increases were observed in expression levels of AC133 and VEGFR-2 but VE-cadherin levels remained constant. A statistically significant increase was seen in the expression of Tie-2 in the infiltrating carcinoma relative to the control group.

DISCUSSION

Our findings demonstrate a clear increase in peripheral Tie-2 expression in breast carcinoma patients with a smaller increase in AC133 and VEGFR-2 expression in these patients. Possible reasons for this difference may include variations in EPC marker expression at the stage of development in which they were studied, namely in the peripheral circulation following release from the bone marrow but prior to homing in on the tumor site. Recent studies have shown that the Tie-2 ligand potently stimulates EPC release in animal models.[8] Our data suggest that Tie-2 levels may be the easiest to use as markers of vasculogenesis in breast cancer. While these data suggest a role for EPC in the process of tumor vasculogenesis, additional studies which incorporate other modalities such as flow cytometry and immunohistochemistry, as well as other involved tissues such as bone marrow and tumor tissue, are needed to better define the properties and functions of this cell population. Additionally, Tie-2 expression in the blood of cancer patients may be a much-needed and tractable surrogate marker for anti-angiogenesis therapy.

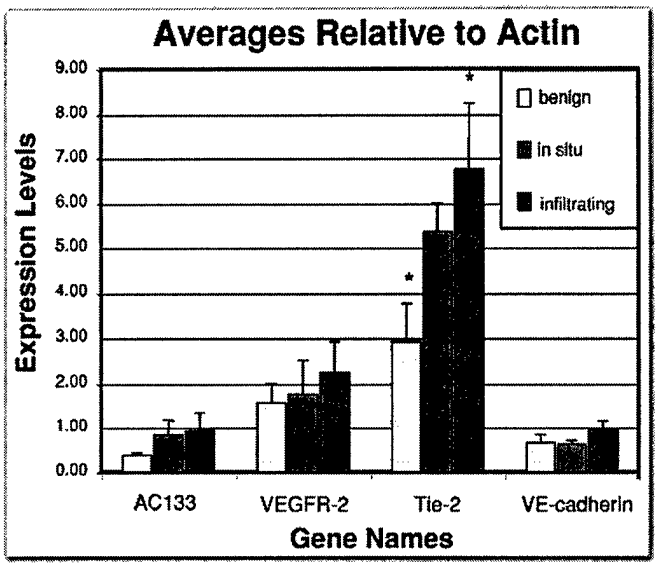

Figure 1: Relative expression levels of EPC markers increase in the blood of breast cancer patients. Quantitative RNA levels by real-time RT-PCR are presented relative to Actin and grouped by tumor histology. *p = 0.038.

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Research Paper

Inhibition of Tie-2 Signaling Induces endothelial Cell Apoptosis, Decreases Akt Signaling, and Induces Endothelial Cell Expression of the Endogenous Anti-Angiogenic Molecule, Thrombospondin-1

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KEY WORDS

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ABSTRACT

Small molecule inhibitors of endothelial cell specific tyrosine kinases are currently under investigation as potential means to block tumor angiogenesis. We have investigated the utility of blocking Tie-2 signaling in endothelial cells as a potential anti-angiogenic strategy. We have found that interruption of Tie-2 signaling either via RNAi or overexpression of a kinase-dead Tie-2 led to loss of endothelial cell viability, even in the presence of serum. Mechanistically, this is linked to a block in Akt signaling and increased thrombospondin expression. Thrombospondins are endogenous anti-angiogenic matricellular proteins known to regulate tumor growth and angiogenesis. We observed that both Tie-2 and subsequent PI3Kinase signaling regulates thrombospondin-1 expression. These data have lead to the model that Angiopoietin signaling through Tie-2 activates PI3Kinase/Akt, which represses thrombospondin expression. Thus, targeting Tie-2 with small molecules maybe efficacious as an anti-angiogenic therapy.

INTRODUCTION

Angiogenesis, the formation of new capillaries from pre-existing blood vessels, is a fundamental process required for normal embryonic development and for the development of pathological conditions such as cancer.[1-3] There are two major families of endothelial receptor tyrosine kinases that are critical for vascular development, the vascular endothelial growth factor (VEGF) receptors, VEGFR-1 and VEGFR-2, and Tie-1 and Tie-2 receptors.[4-8] The Tie-2 tyrosine kinase receptor is largely specific for the vascular endothelium and the ligands for this receptor is a family of proteins called Angiopoietins.[6,9] One current view of the functional properties of the angiopoietin/Tie-2 system is that in the quiescent state of the vasculature, Ang1, which is constitutively expressed in many tissues, acts on the endothelial cells (ECs) by activating Tie-2 , resulting in the maintenance of cell-cell contacts between ECs and pericytes, thereby stabilizing the blood vessel.[10-12] In addition, recent studies using inducible transgenics to overcome the earliest developmental phenotypes of the Tie-2 null animals clearly shows that Tie-2 function is critical for endothelial cell survival at later stages.[13] For neovascularization to take place in a mature tissue, destabilization of the blood vessels is thought to be induced by blocking Tie-2 signaling through binding of antagonistic Ang2.[14] The further fate of these vessels is dependant on the presence or absence of endothelial survival factors such as VEGF and PlGF.[15-17]

Investigators have used soluble Tie-2 ligand binding domains to block endogenous Tie-2 activation, angiogenesis and tumor growth in murine tumor models.[18] However, these approaches block ligand-binding rather than receptor function. Some literature on the Angiopoietins has suggested that there may be signaling and nonsignaling components to their functions (reviewed in ref. 12 and 19). One example of a nonsignaling function was reported by Carlson et al., who reported that angiopoietins may mediate integrin adhesion.[20] This raises the possibility that small molecule inhibitors of Tie-2 that block only the signaling function may not fully recapitulate approaches designed to block ligand binding. Reports of Tie-2 kinase inhibitors as anti-cancer therapeutics[21-23] suggest that loss of kinase activity may by a viable anti-angiogenic therapeutic. Given the possibility of nonspecific targets with such inhibitors, we sought to genetically validate the Tie-2 kinase as a target for anti-angiogenic therapy. We initiated a study to look at Tie-2 signaling that mediates endothelial cell survival, comparing both total loss of function and loss of kinase function approaches. These different approaches could have alternate phenotypes if nonsignaling functions were important to endothelial cell survival. We performed a series of experiments using both RNAi and overexpression of a kinase-dead mutant Tie-2 protein in primary dermal microvascular endothelial cells. We observed that loss of Tie-2 by either means induced apoptosis and thrombospondin-1 expression in endothelial cells.

Thrombospondin-1 is a matricellular protein that can induce endothelial cell apoptosis.[24] This function is in part due to activation of caspases.[25] These studies suggest that blocking Tie-2 signaling induces TSP-1 in endothelial cells where it may have profound autocrine effects on endothelial cell viability.

MATERIALS AND METHODS

Cell Culture. Primary human microvascular endothelial cells were isolated as previously described[26] and grown in EGM-2 MV medium (Cambrex, MD) on plates coated with 30 μg/ml Vitrogen 100 (Cohesion, CA). Passage 4–6 cells were used for studies.

pSuper Vector-Based RNAi System. pSuper vector was provided by Dr. Reuven Agami (Division of Molecular Carcinogenesis, the Netherlands Cancer Institute).[27] Human Tie2 RNAi oligos were designed and synthesized by Oligoengine Company (Oligoengine, WA). Four pairs of Tie2 oligonucleotides were synthesized. The sequences are:

T2.1 Forward
5'GATCCCCAGCTTCTATCGGACTCCCTTCAAGGAGGGAGT CGATAGAAGCTGTTTTTGGAAA

T2.1 Reverse
5'AGCTTTTCCAAAAACAGCTTCTATCGGACTCTCTCTTGA-AGGGAGTCCGATAGAAGCTGGGG

T2.2 Forward
5'GATCCCCGGTGCCATGGACTTGATCTTTCAAGAGAAGAA-CAAGTCCATGGCACCTTTTTGGAAA

T2.2 Reverse
5'AGCTTTTCCAAAAAGGTGCCAGACTTGATCTTCTCTTGA-AAGAACAAGTCCATGGCACCGGG

T2.3 Forward
5'GATCCCCGGCTTGTGAACTGCACACGTTCAAGAGACGT-GTGCAGTTCACAAGCCTTTTTGGAAA

T2.3 Reverse
5'AGCTTTTCCAAAAAGGCTTGTGAACTGCACACGTCTCTT-GAACGTGTGCAGTTCACAAGCCGGG

T2.4 Forward
5'GATCCCCGATGCGTCAACAAGCTTCCTTCAAGAGAGGAA-GCTTGTTGACGCATCTTTTTGGAAA

T2.4 Reverse
5'AGCTTTTCCAAAAAGATGCGTCAACAAGCTTCCTCTCTTG-AAGGAAGCTTGTTGACGCATCGGG

Table 1. **PRIMER SEQUENCES**

Gene	Primer sequence
Mouse Tie2	Forward 5'- TCCTGTGCTTGACTGGAATG-3'
	Reverse 5'- CGAATAGCCATCCACTATTGTCC-3'
Mouse TSP-1	Forward 5'- TAACGGTGTGTTTGACATCTT-3'
	Reverse 5'- TGGATAGATCTTGGCCCTTCA-3'
Mouse GAPDH	Forward 5'-GGCAAATTCAACGGCACAGT -3'
	Reverse 5'-AAGATGGTGATGGGCTTCCC -3'

All sequences were blasted to the genomic database to make sure they had no significant homology with other genes. Synthesized oligonucleotides were dissolved into water and mixed with annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH pH 7.4, 2 mM Mg-acetate). After incubating at 95°C for 4 minutes and 70°C for 10 minutes, annealed oligonucleotides were slowly cooled to 4°C. Annealed oligonucleotides were incubated with T4 PNK at 37°C for 30 minutes to phosphorylate them and the enzyme was inactivated by incubation at 70°C for 10 minutes. The phosphorylated oligonucleotides were ligated into *Bgl*II, *Hind*III (NEB, MA) sites of the pSuper vector. The positive clones of Tie2-pSuper RNAi plasmid DNA were confirmed with *EcoRI-Hind*III digestion.

Nucleofector Transfection. Dermal microvascular endothelial cells were grown on flasks precoated with Vitrogen in EGM-2 MV complete medium. After 4 days, the cells were harvested with trypsin-EDTA and washed. For each 1 x 10^6 cells, 100 μL Nucleofector (Amaxa Biosystems, Germany) with 2 μg DNA was used. The samples were transferred into Amaxa certified cuvettes (Amaxa Biosystems, Germany) and program S-05 was used. Endothelial cells transfected with RNAi or Tie-2 plasmids were then removed from the cuvette and plated into a precoated six-well tissue culture plate for 48 hours for further analysis.

Western Blots. Endothelial cell lysate were collected with RIPA buffer (Boston Bioproducts, MA) and protein concentrations were quantified with Bio-Rad DC Protein Assay Reagents (Bio-Rad, CA). 20 μg protein samples were run on 10% SDS-PAGE and transferred to nitrocellulose membrane (Bio-Rad, CA). The membranes were blocked with 5% nonfat milk and blotted with Anti-phospho Akt (Pharmingen, CA), Anti-PARP (Upstate Biotechnology, NY), Anti-Tie2 (Upstate Biotechnology, NY), Anti-human PTEN (Cascade Bioscience, MA), Anti-human TSP-1 monoclonal antibody MAI,[28] and anti-α-tublin (Sigma, MO) antibodies. Pierce detection reagents (Pierce Technology, IL) were used to visualize HRP-labeled secondary antibodies.

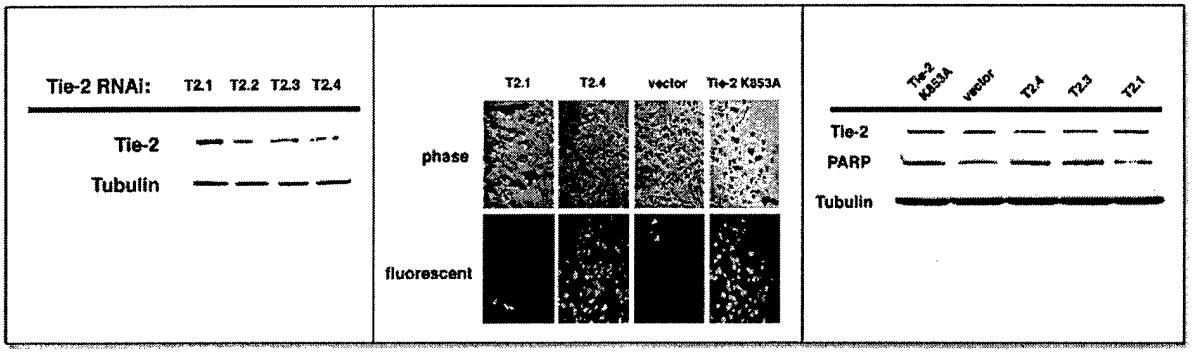

Figure 1. Loss of Tie-2 function induces endothelial cell apoptosis (A) Western blot analysis of Tie2 expression in human dermal microvascular endothelial cells transfected with Tie-2 RNAi's. (B) In Situ staining of activated caspases in endothelial cells transfected with Tie-2 RNAi and Kinase-dead Tie-2 (C) Western blot analysis of cleaved PARP in human dermal microvascular endothelial cells transfected with Tie-2 RNAi and Kinase-dead Tie-2.

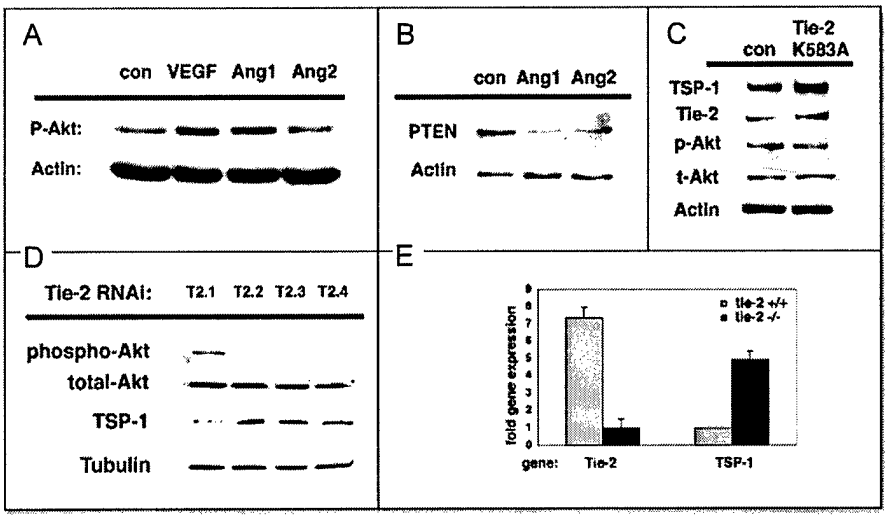

Figure 2. Signaling through Tie-2 mediates PI3Kinase regulation and thrombospondin expression. (A) 15 minute stimulation with VEGF-A and Ang 1 induced Akt phosphorylation in endothelial cells. (B) Overnight exposure to Ang1 and Ang2 decrease PTEN expression in endothelial cells. (C) Overexpression of kinase-dead Tie-2 leads to steady-state decreases in phosphorylated Akt, and increases in TSP-1. (D) Steady-state levels of phosphorylated-Akt are decreased when Tie-2 RNAi blocks Tie-2 expression. (E) Real time PCR analysis of TSP-1 and Tie2 RNA expression in Tie2 knockout and wildtype mice.

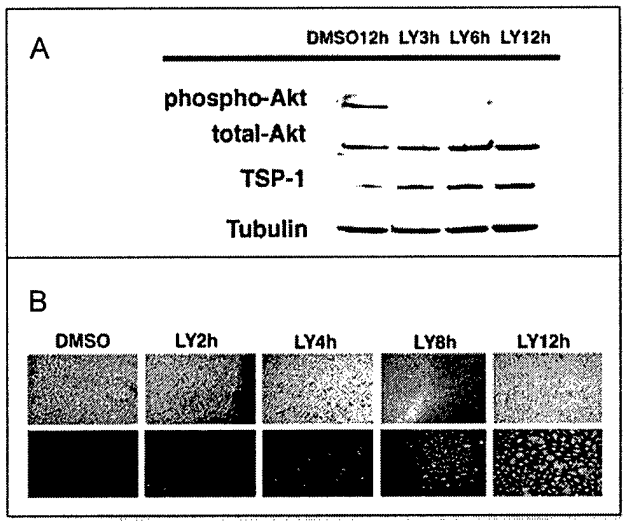

Figure 3. PI3 Kinase signaling mediates expression o f TSP-1 and caspase activation in primary endothelial cell culture. (A) Western blot analysis of TSP-1 and Akt expression and (B) activated caspases in human dermal microvascular endothelial cells incubated with LY 294002 at 25 μM from 2–12 hours.

Analysis of Caspase Activation. To determine whether human dermal microvascular endothelial cells were undergoing apoptosis after Tie2 RNAi transfection or incubation with LY 294002 (Calbiochem, CA) or a recombinant version of all three type' I reeats of TSP-1(8TSR), we incubated endothelial cells transfected with Tie2 RNAi or treated with LY 294002 at 25 μM or 3TSR at 100 μg/ml with CaspACE-FITC-VAD-FMK In Situ Marker (Promega, WI) at 10 μM for 20 minutes, which binds activated caspases.[29] Images were captured using the Lecia DC200 digital camera and imported into Adobe Photoshop 5.0.

Real time quantitative reverse transcription-polymerase chain reaction (QRT-PCR). Total RNA was isolated from freshly homogenized Tie2 knockout and Tie2 wildtype embro tissue as well as endothelial cells with RNeasy Mini Kit (Qiagen, CA) according to manufacturer's protocol. 2 μg of total RNA was used for reverse transcription with TaqMan Reverse Transcription Reagents Kit (Applied Biosystems, CA) according to the manufacturer's instructions. PCR primers were synthesized by Integrated DNA Technologies, Inc. (Integrated DNA Technologies, Inc., IA). Real time PCR reactions were conducted in 25 μl reaction volume containing 1 μl of cDNA and 12 μl 2 X Sybr Green Master Mix (Applied Biosystems, CA). PCR mixtures were preincubated at 50°C for 2 minutes, then 95°C for 10 minutes followed by 40 cycles of two-step incubations at 95°C for 15 seconds and 60°C for 1 minutes with ABI PRISM 7700 Sequence Detection System (Applied Biosystems, CA). For each set of primers, specific standard curve was established to calculate the specific gene expression level normalized to GAPDH expression. Specific gene expression change was calculated by comparing the gene expressions normalized to GAPDH in different groups according to the manufacturer's instructions. Primer sequences are listed in Table 1.

RESULTS AND DISCUSSION

We compared endothelial cell viability after loss of total Tie-2 expression via RNAi to that of loss of kinase activity using overexpression of a kinase-dead Tie-2 protein.[8] Four RNAi sequences were designed and cloned into the pSUPER vector for transient expression following transfection. Three of the four sequences (T2.2-T2.4) were effective at reducing endogenous Tie-2 levels (Fig. 1A). The fourth RNAi (T2.1), which did not reduce Tie-2 expression, was used as a negative control in future experiments. Both blocking Tie-2 expression with RNAi and overexpression of a kinase-dead allele compromised endothelial cell viability in serum-containing media. To determine whether loss of viability was due to induction of apoptosis, we assayed for caspase activity and PARP cleavage. Figure 1B shows strong induction of caspases using a fluorescent Caspase substrate assay (CaspACE-FITC-VAD-FMK In Situ staining of activated caspases). Caspase induction was comparable between RNAi treated and Kinase impaired cultures. Similarly, both approaches induced PARP cleavage suggesting that indeed, even in serum-containing media, loss of Tie-2 signaling in endothelial cells was sufficient to cause apoptosis (Fig. 1C).

The work of others has shown that Ang1 activates PI3Kinasee and Akt phoshorylation via Tie-2 (18-20). We reproduced this result in our system and saw increased Akt phosphorylation following 25 ng/mL VEGF-A and 50ng/mL Ang1 but not 50ng/mL Ang2 stimulation (Fig. 2a). However, overnight exposure to both Ang1 and Ang2 reduced PTEN expression, suggesting that there may be both transient links to Akt signaling and more sustained effects via

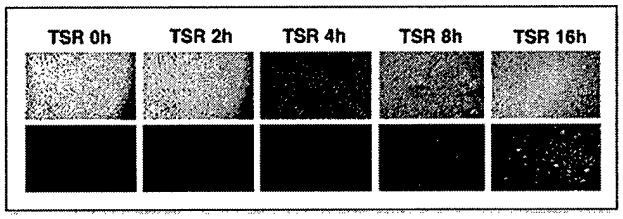

Figure 4. 8-16 hours of treatment with recombinant type I repeats from TSP-1 induce caspase activation. In Situ staining of activated caspases in human dermal microvascular endothelial cells incubated with 3 TSRs at 100 μg/ml show similar result on endothelial cell caspase activation as LY 294002 but with slightly delayed kinetics.

PTEN (Fig. 2b). When we blocked Tie-2 signaling with the kinase-dead mutant, we saw that basal levels of Akt phosphorylation were reduced in serum-containing media (Fig. 2c). In addition, we observed increased expression of TSP-1. We observed similar results following RNAi transfection (Fig. 2d). Additional support for the hypothesis that TSP-1 is regulated by Tie-2 signaling was obtained from the Tie-2 null mice. We compared *tsp-1* mRNA levels in Tie-2 null and wildtype embryos using real time quantitative RT-PCR and found that *tsp-1* levels were increased in null embryos (Fig. 2e). Genotyping of the embryos was further confirmed by RT-PCR of *tie-2*.

While we saw both effects on Akt phosphorylation and TSP-1 expression following loss of Tie-2 function, we did not know whether these two observations were mechanistically linked. To test this, we used the LY 294002 inhibitor to block PI3 Kinase activity in endothelial cell culture for 3,6 and 12 hours. We observed loss of Akt signaling by 3 hours and a gradual increase in TSP-1 levels from 3-12 hours (Fig. 3). We also observed induction of caspases after 8 hours of LY treatment. This suggests that sustained inhibition of PI3Kinase activity will both induce apoptosis and TSP-1 expression in endothelial cells.

Previous investigators have shown that the type I repeats of TSP-1 are capable of anti-angiogenic activity via apoptosis.[30] In our caspase induction assay, we observed that recombinant type I repeats of TSP-1 (designated 3TSR), recapitulated the activation of caspases between 8-12 hours, similar to the effect of Tie-2 blockade and with slightly delayed kinetics compared to the LY treatment (Fig. 4). Together, these data support the hypothesis that kinase inhibition of Tie-2 may be effective at reducing endothelial cell viability, in part by blocking PI3Kinase activity which subsequently induces TSP-1 autocrine signaling. Both loss of PI3Kinase activity and induction of TSP-1 can cause endothelial cell apoptosis.

While PI3Kinase regulates cell survival via several downstream pathways, these findings suggest that inhibition of Tie-2 signaling, even in full media with serum, abrogates this pathway and that at least one downstream mediator of the ensuing apoptosis is autocrine TSP-1 expression. Thrombospondins are negative regulators of angiogenesis that can induces apoptosis (27,39) and TSP-1 expression is correlated to a good prognosis in human tumors.[31-37] According to the hypothesis that angiogenesis is modulated by a balance in pro- and anti- angiogenic molecules, the Tie-2 inhibitors may inhibit tumor growth by shifting the balance of gene expression in favor of anti-angiogenic molecules.

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